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Regulation of triggering receptor expressed on myeloid cells-2 (TREM2) expression in microglia

Contents

Abstract

Background: Activation of microglia is a feature of several neurodegenerative diseases. TREM2 is an immune receptor expressed by macrophages and microglia, which negatively regulates immune responses of these cells. Microglia-associated brain inflammation may be alleviated by up-regulation of microglial TREM2. However, it is unclear whether regulation of TREM2 expression in microglia is consistent with classical (M1) and alternative (M2) activation pathways of macrophages. **Aims:** To determine if microglial expression of TREM2 and its adaptor protein DAP12 is regulated by M1 or M2 activation. **Methods:** BV2 microglial cells were stimulated by LPS, IL-4, or HMGB1, respectively, with PBS as control. 6-hour and 24-hour stimulations were applied to each treatment. Expression of TREM2, DAP12, iNOS, IL-1β and Arg-1 was analysed by quantitative real-time PCR. Relative quantification of results was performed using delta delta Ct method. One-sample Student's t-test was used to detect difference between treatment and control. **Results:** Induced polarisation toward M1 and M2 activation phenotypes were confirmed in microglia. TREM2 was down regulated by LPS (Mean ± SD: 0.95±0.56 for 6h and 0.40±0.07 for 24h) and up regulated by IL-4 (6h: 4.36±2.99; 24h: 1.74±0.68) and HMGB1 (6h: 4.56±4.20; 24h: 1.20±0.20). DAP12 presented no obvious difference between treatments. **Conclusion:** Microglia are subjected to M1 and M2 polarisation. M2 cytokine IL-4 up regulates both TREM2 and Arg-1, which attenuate brain inflammation. Regulation of microglial TREM2 provides a promising target for treatment of neurodegenerative diseases.

1 Introduction

1.1 Triggering Receptor Expressed on Myeloid Cells-2 (TREM2)

1.1.1 Biology of TREM2

Triggering receptors expressed on myeloid cells (TREMs) are cell surface receptors of immunoglobulin superfamily (Ig-SF). They are expressed primarily although not exclusively by cells of myeloid lineage. They were first identified by Bouchon and colleagues in neutophils and monocytes (Bouchon et al., 2000). The human TREM gene cluster is located at chromosome 6 and a TREM-related cluster was defined on mouse chromosome 17 (Daws et al., 2001). Two isoforms, TREM1 and TREM2, have been identified in humans. Orthologues of human TREM1 and TREM2 are expressed in mice as well as functional TREM3, which is a pseudogene in humans.

TREMs are transmembrane glycoproteins consisting of an Ig-SF V-type extracellular domain, a transmembrane domain charged by lysine residue, and a short cytoplasmic domain without motifs for docking of signalling mediators. The positively charged transmembrane domain of TREM2 facilitates its association with adaptor DAP12, whose transmembrane domain contains a negatively charged aspartic acid residue (Daws et al., 2001). The cytoplasmic domain of DAP12 contains immunoreceptor tyrosine-based activation motifs (ITAMs). After being phosphorylated by the downstream signalling from DAP12, ITAMs recruit protein tyrosine kinases, initiating a sequential of phosphorylation events to regulate cellular activities.

1.1.2 Functions of TREM2

TREM2 has been identified on a variety of cells in myeloid lineage, including dendritic cells (DCs) (Bouchon et al., 2001), macrophages (Daws et al., 2001), microglia (Schmid et al., 2002) and osteoclasts (Paloneva et al., 2003). It contributes to both non-immune and immune functions of these cells. The non-immune function is demonstrated by patients with Nasu-Hakola disease, also known as polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL)

(Paloneva et al., 2003; Paloneva et al., 2002), which is characterised by pre-senile dementia and bone cysts and caused by loss-of-function mutations in the TREM2 and/or DAP12 genes. Abnormalities in osteoclasts and microglia have been proposed to underlie the pathological features of the disease. The role of TREM2 in immune system is reflected by its relatively high expression on immuno-phagocytic cells[i]. Whereas TREM1 is a potent amplifier of pro-inflammatory responses (Bouchon et al., 2001), TREM2 is a negative regulator of immune responses. Previous studies revealed that TREM2 attenuated inflammatory activities of macrophages (Daws et al., 2001; Takahashi et al., 2005). TREM2- and/or DAP12- deficit macrophages have increased production of inflammatory cytokines, such as interferon-gamma (IFNγ) and inducible nitric oxygen synthase (iNOS) (Hamerman et al., 2005), and transduction of TREM2 led to reduced expression of them (Takahashi et al., 2005). An *in vivo* study in animal models of multiple sclerosis claimed that TREM2 expression on microglia was enhanced during progression of the disease and blockade of TREM2 exacerbated the clinical symptoms (Ford and McVicar, 2009). These data suggest that modulation of TREM2 expression or function may provide novel insights into regulation of CNS inflammation.

1.2 Macrophages in Immune Responses

Macrophages are important cells in immune defence, tissue homeostasis and repair (Mosser and Edwards, 2008). They are highly versatile cells that can sense and respond to a huge variety of external signals. Accordingly, the activation of macrophages comprises a broad spectrum of responses. At each end of this spectrum, the phenotype of macrophage activation is broadly classified as classical or alternative, each of which is associated with distinct functional properties and profiles of activating cytokines (Classen A et al., 2009).

[i] Generally, DCs are phagocytes that initiate immune responses and are present in spleen, lymph nodes, skin and mucosal surfaces. The phagocytes circulating in peripheral blood are monocytes, while those tissue-resident phagocytes are known as macrophages. There are some tissue-specific macrophages, such as osteoclasts in bone and microglia in the CNS.

1.2.1 Classical Activation of Macrophages (M1)

Classical activation of macrophages is typically associated with stimulation by Toll-like receptor (TLR) ligands such as lipopolysaccharide (LPS) and/or the Th1 cytokine, IFNγ. These macrophages are important components of immune defence and inflammatory responses. Macrophages recognise invading pathogens via TLRs and produce iNOS. The latter facilitates synthesis of nitric oxide (NO) from L-arginine for pathogen killings. Classically activated macrophages also mediate Th1 immune responses by secreting pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNFα). Macrophages adopting this phenotype are commonly known as type-I activated macrophages (M1), which are highly microbicidal and pro-inflammatory.

1.2.2 Alternative Activation of Macrophages (M2)

Th2 cytokines-induced inflammatory macrophages exhibit an alternative activation phenotype distinct from that of M1 macrophages. These macrophages are, therefore, known as alternatively activated macrophages, or type-II activated macrophages (M2). They are characterised by enhanced capacity of phagocytosis, increased expression of MHC class II antigen, and reduced production of pro-inflammatory cytokines (Stein et al., 1992). Alternatively activated macrophages produce arginase-1 (Arg-1) to metabolise L-arginine into polyamines and proline, which competes with iNOS for L-arginine and thus can prevent nitric oxide release. Polyamines are involved in cell growth and proliferation and proline is a component of collagen. In alternative activation, therefore, tissue repair is promoted whereas inflammatory responses are precluded.

1.2.3 Regulatory Macrophages

Some researchers described a third population of macrophages characterised by high production of IL-10 (Mosser and Zhang, 2008). These cells contribute to immune regulation of macrophage activities. Similar to classically activated macrophages, this population are induced by TLR stimulation but with different priming signals. These

activated macrophages exhibit increased production of iNOS but not Arg-1 (Edwards et al., 2006). Since these macrophages share the same activating stimulus (LPS) and immune product (iNOS) with M1 macrophages, they were not distinguished as a third population apart from M1 and M2 macrophages in the present study.

1.3 Microglia, the Resident Macrophages in the Central Nervous System (CNS)

1.3.1 Immune Activities of Microglia

As a specialised subtype of macrophages, microglia are CNS-resident immune cells capable of phagocytosis and cytokine production. Their immune function is regulated by multiple signalling pathways that collectively determine the activation state. As surveillance cells, microglia play a dual role in the CNS. These phagocytes eliminate invading pathogens, apoptotic cells and tissue debris during neuropathology thus ameliorate inflammatory damages to brain tissues. On the other hand, they generate numerous cytokines and enzymes to mediate immune responses and brain inflammation. The balance of pro-inflammatory and anti-inflammatory activities of microglia is crucial for brain homeostasis, which is determined by a number of intracellular pathways that transduce cell surface signals. An important example is the interplay between ITAM- and immunoreceptor tyrosine-based inhibition motif (ITIM)-mediated pathways. Dysfunction of microglia is a common characteristic of brain diseases, such as multiple sclerosis, Alzheimer's disease and brain ischaemia, *et al*. It is reported that regulation of over-activated microglia is a potential treatment strategy for these neurodegenerative pathologies (Osherovich, 2010; Takahashi et al., 2007; Yenari et al., 2010).

1.3.2 TREM2-mediated Regulation of CNS Inflammation

As described above, TREM2 is a negative regulator of immune responses of macrophages and recent evidence suggests microglial TREM2 restrains tissue-damaging inflammation in the CNS. Accordingly, it is important to understand how microglial expression of TREM2 is regulated. Expression of TREM2 in macrophages is

attenuated by LPS stimulation and is enhanced in some macrophage subpopulations by exposure to IL-4 (Turnbull et al., 2006), but it is unclear whether similar pattern of TREM2 expression occurred in microglia.

LPS is a component of gram-negative bacterial cell walls, and therefore mimics the classical activation of macrophages associated with bacterial infection. Non-microbial activation of macrophages is also important in the context of sterile inflammatory conditions such as ischaemia and trauma. In these conditions, molecules released from necrotic cells, referred to as damage-associated molecular patterns (DAMPs) are detected by macrophages/microglia. One example is high-mobility group box-1 (HMGB1), a pro-inflammatory cytokine (Ito et al., 2007) strongly implicated as a necrosis-induced DAMP, particularly in the brain.

iNOS and IL-1β are potent mediators of immune defence of macrophages, and Arg-1 is important in tissue repairing. In reference to classical and alternative activation of macrophages, iNOS and IL-1β were chosen as indicators for M1 activation and Arg-1 as a signature marker for M2 activation of microglia.

2 Aims

The primary aim of this study was to determine if expression of TREM2 and its adaptor protein DAP12 is regulated in the microglial BV2 cell line by classical or alternative activation or HMGB1, a representative DAMP. Classical activation was induced by LPS and alternative activation by IL-4. To validate the expected polarisation of microglia towards an M1 or M2 phenotype, the expression of the M1 genes iNOS and IL-1β and the M2 gene Arg-1 was also measured in the experiment.

3 Materials and Methods

3.1 Materials

3.1.1 Cells

The BV2 cell line is a murine microglial culture immortalised by infection with a

v-raf/v-myc recombinant retrovirus (Blasi et al., 1990). As they exhibit some properties of reactive microglia (phagocytic abilities and production of cytokines), BV2 cells have been extensively used as an *in vitro* culture system for microglia in published studies (Brautigam et al., 2005; Raouf et al., 2007; Watters et al., 2002).

3.1.2 Reagents

LPS and HMGB1 were used as M1-stimulating reagents, and IL-4 for M2-activation. LPS from *Escherichia coli* (serotype O127:B8) was purchased from Sigma-Aldrich Co., recombinant mouse IL-4 from R & D Systems Inc., and HMGB1 from HMGBiotech Srl. Dulbecco's Phosphate Buffer Saline (PBS, Life Technologies) was used as the vehicle control.

3.2 Methods

3.2.1 Culturing and Passaging of BV2 Microglial Cells

BV-2 cells were grown in Dulbecco's Modified Eagle Media (DMEM, 500ml media with glucose 4500mg/L, L-glutamine, $NaHCO_3$ and pyridoxine.HCl; Sigma) supplemented with 5% heat inactivated fetal calf serum (FCS, Sigma), 1% penicillin G and 1% streptomycin. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Media was changed completely every three days. Successive cell passages were performed by incubating cells in 0.4mM trypsin (2ml per 75T flask) at 37°C for 30 seconds and detached cells by patting flasks. 6ml DMEM was added to inhibit trypsin. For 1:2 splitting, half of the cell suspension was transferred into a new flask and was resuspended with 8ml fresh DMEM with 5% FCS, 1% penicillin and 1% streptomycin.

3.2.2 Immunocytochemistry

BV2 cells were plated at 50,000 cells per well and incubated at 37°C in 5% CO_2 for 24 hours. Cells were thoroughly washed with PBS and then fixed with 4% paraformaldehyde (PFA) for 30 minutes. After fixation, cells were stored at 4°C in PBS for one week. Cells were permeabilised by 0.1% Triton-X100 (Sigma) at room temperature for 5 minutes and washed with PBS. 0.25% NH_4Cl was used to quench autoflurescence. The non-specific binding sites were blocked with 5% mixed donkey and goat serum in blocking buffer (0.1% Bovine Serum Albumin Protein in PBS) for one hour.

Ionized calcium binding adaptor molecule 1 (Iba1) was used as a marker of microglia (It is a protein specifically expressed by macrophages/microglia and is up regulated during the activation of these cells). BV2 cells were double-stained with either TREM2 and Iba1, or DAP12 and Iba1. Monoclonal antibodies (Abs) for immunostaining were purchased from R & D systems. Cells were incubated with the primary antibodies (Table 1) at room temperature for one hour and then washed with blocking buffer. The following procedures were conducted in the dark. Cells were incubated with fluorescence-coupled secondary antibodies (Table 1) at room temperature for one hour then washed with PBS. For counter staining, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, 1:1000, Sigma) for 5 minutes. Cells were washed and stored in PBS at 4°C in the dark for 48 hours. Fluorescence images were collected at 630X magnification by confocal laser scanning microscopy (LSM 710, Zeiss). Images were processed with ZEN 2009 Light Edition.

Table 1: Antibodies for immunocytochemistry.

Staining targets	**Primary antibodies**			**Secondary antibodies**		
	Abs	Species	Dilution	Abs	Species	Dilution
TREM2	Anti-TREM2	rat	1:100	Anti-rat-568	goat	1:1000
DAP12	Anti-DAP12	goat	1:100	Anti-goat-594	donkey	1:1000
Iba1	Anti-Iba1	rabbit	1:250	Anti-rabbit-488	donkey	1:1000

3.2.3 Stimulation of BV2 Cells

BV2 cells were seeded at 250,000 cells per well to 12-well plates. After incubation at 37°C for 24 hours, media was replaced with 1ml serum-free DMEM supplemented with penicillin and streptomycin. After incubation for two hours, cells were stimulated with 1µg LPS, 20ng IL-4 or 100ng HMGB1, or PBS as control. Doses were selected based on previous experiments in the group. After incubation with the stimulating reagent at 37°C for 6 hours or24 hours, media was aspirated and replaced with PBS to wash cells. To precipitate RNA, 0.5ml per well TRIzol reagent (Life Technologies) was added for 2.5 minutes and, meanwhile, homogenised cell lysate by pipetting. Cell lysate was transferred to fresh tubes and stored at -70°C.

3.2.4 RNA Extraction and Purification

3.2.4.1 RNA Isolation from Stimulated BV2 Cells

The cell lysate was thawed from -70°C. The amounts of reagents used in the following procedures were for 0.5ml TRIzol-suspended cell lysate per tube. 100µl 1-Bromo-3-Choloropropane (BCP, Sigma) was added and incubated at room temperature for three minutes. Centrifuged at 12,000*Xg* at 4°C for 15 minutes to extract RNA into the upper translucent aqueous layer. Transferred the aqueous layer to a fresh tube. Added 100µl BCP to the aqueous layer and repeated the above steps of incubation and centrifuge. Added 1µl neat linear acrylamide (Life Technologies) and 250µl Isopropanol (Sigma) to precipitate RNA. Centrifuged at 12,000*Xg* at 4°C for 10 minutes. Then removed the supernatant completely and washed RNA pellet with 500µl 75% ethanol. Centrifuged at 7,500*Xg* at 4°C for 5 minutes. Removed supernatant completely and left the pellet to air dry until it became translucent. Resuspended the pellet with 40µl nuclease-free water (the volume depends on the size of pellet) and homogenised by pipetting. Then incubated at 55°C for 10 minutes and placed the tubes on ice.

3.2.4.2 DNase Treatment

DNA-free kit (Life Technologies) was applied to remove residual genomic DNA that might be contained in RNA samples. For 40µl RNA suspension, 4µl (10% volume) 10XDNase I Buffer (100mM Tris-HCl pH 7.5, 25mM MgCl2, 5mM CaCl2) and 1µl recombinant DNase I (2U/µl) were added and incubated at 37°C for 25 minutes. Then 4µl (10% volume) resuspended DNase inactivation reagent was added and incubated at room temperature for 2 minutes. Centrifuged at 10,000*Xg* at room temperature for 1.5 minutes and transferred the upper aqueous layer to a fresh tube. RNA concentration and purity were measured in NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies).

3.2.5 cDNA Synthesis by Reverse Transcription

Reverse transcription of RNA was performed with Oligo $(dT)_{15}$ primers and SuperScript III recerse transcriptase. Mastermix 1 was prepared for RNA samples extracted from cells with each treatment (LPS, IL-4, HMGB1 or PBS): 2µg RNA sample, 2µl Oligo $(dT)_{15}$ primers (0.5µg/µl, Promega), 2µl dNTP (10mM, Invitrogen), and topped up with nuclease-free water to 26µl. Incubated mastermix 1 in PCR thermal cycler T100 (BIO-RAD) at 65°C for 5 minutes to denature the secondary structures of RNA potentially formed in samples, and chilled on ice for 2 minutes. While matermix 1 was being incubated, mastermix 2 was prepared: 8µl 5X First Strand Buffer (Invitrogen), 4µl Dithiothreitol (DTT, 0.1M, Invitrogen), 1µl RNasin Plus RNase inhibitor (40U/µl, Promega) and 1µl SuperScript III recerse transcriptase (200U/µl, Invitrogen). The mixture of mastermix 1 and Mastermix 2 was incubated at 50°C for 50 minutes (for reverse transcription) and 70°C for 15 minutes (for inactivation of reverse transcriptase). Concentration and purity of the resulting cDNA was measured by NanoDrop and then stored at -20°C.

3.2.6 Quantitative Real-time PCR (qRT-PCR) Analysis for Expression of TREM2, DAP12 and Inflammatory Gene Transcripts

3.2.6.1 Selection of Reference Genes

In order to select reference genes that are stable under the experimental conditions, geNorm^{PLUS} analysis (http://www.biogazelle.com/genormplus) was performed by inputting information of samples (two for LPS, IL-4 and HMGB1 each) and choosing five candidate reference genes (18S rRNA, YWHAZ, Actin beta, GAPDH and B2M).

3.2.6.2 Optimisation of Primers for Target Genes and Reference Genes

Purified primers for target genes were generated (Invitrogen, Life Technologies) and optimised to an equal annealing temperature 60°C. Sequences of the first set of primers (Table 2) were retrieved from literature (Edwards et al., 2006; Kiialainen et al., 2005). Before performing experiment with BV2 cells, primer efficiencies were tested in splenocytes for TREM2 expression, with one M1 indicator iNOS, one M2 indicator Arg-1 and one reference gene GAPDH. Ten-fold serial dilutions of cDNA were made (1000ng, 100ng, 10ng, 1ng, and 0.1ng). Primers efficiencies of both target genes and reference genes were tested by serial dilution qRT-PCR. Standard curves were constructed by the Ct (y-axis) versus log cDNA dilution (x-axis). The primer efficiency (E) of one cycle in the exponential phase was calculated according to the equation: $E=10^{-(1/slope)}-1$ (Pfaffl, 2001). Poor specificity and efficiencies were obtained with the first set of primers (Table 2) which was likely because of the large product sizes.

Table 2: The first set of primers for target genes.

Target genes	Primers	Sequences (5' to 3')	Length of products
TREM2	Forward	ATG GGA CCT CTC CAC CAG TTT CTC C	681
	Reverse	CGT ACC TCC GGG TCC AGT GAG G	
iNOS	Forward	CCC TTC CGA AGT TTC TGG CAG CAG C	497
	Reverse	GGC TGT CAG AGC CTC GTG GCT TTG G	
Arg-1	Forward	CAG AAG AAT GGA AGA GTC AG	250
	Reverse	CAG ATA TGC AGG GAG TCA CC	
GAPDH	Forward	GCA CTT GGC AAA ATG GAG AT	203
	Reverse	CCA GCA TCA CCC CAT TAG AT	

Therefore, a new set of primers (Table 3) were designed using NCBI Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) to produce a shorter product length and tested directly in BV2 cells. Two more genes (DAP12 and IL-1β) were included to validate the induced immune activities of BV2. With the result of geNorm analysis, B2M was used as a second reference gene in addition to GAPDH to provide a better normalisation.

Table 3: The second set of primers for target genes.

Target	Primers	Sequences (5' to 3')	Length of products
TREM2	Forward	CTG CTG ATC ACA GCC CTG TCC CAA	108bp
	Reverse	CCC CCA GTG CTT CAA GGC GTC ATA	
DAP12	Forward	AGG CCC AGA GTG ACA CTT TCC CAA	109bp
	Reverse	GCC AGG GCA ATC AGC AGA GTC A	
iNOS	Forward	GCG GAG TGA CGG CAA ACA TGA C	132bp
	Reverse	AGG TCG ATG CAC AAC TGG GTG AAC	
IL-1β	Forward	CGA CAA AAT ACC TGT GGC CTT GGG C	108bp
	Reverse	TGC TTG GGA TCC ACA CTC TCC AGC	
Arg-1	Forward	GGA GAC CAC AGT CTG GCA GTT GGA	144bp
	Reverse	GGA CAC AGG TTG CCC ATG CAG A	
GAPDH	Forward	TGC ATC CAC TGG TGC TGC CAA	140bp
	Reverse	ACT TGG CAG GTT TCT CCA GGC G	
B2M	Forward	TGG CTC ACA CTG AAT TCA CCC CCA	100bp
	Reverse	TCT CGA TCC CAG TAG ACG GTC TTG G	

3.2.6.3 ROX Reference Dye

ROX (Invitrogen) was the reference dye used to normalise fluorescent reporter signal. The normalised reporter signal was calculated by dividing the reporter signal of SYBR Green I by the reference signal of ROX. The signal was then corrected for inter-well variables within the plate, such as sample amount variation and pipetting inaccuracies.

3.2.6.4 Quantitative Real-time PCR

Each target gene along with reference genes (GAPDH and B2M) and no template control (primer control) was analysed in one plate for qRT-PCR. The qRT-PCR mastermix for each gene was prepared by the following reaction components: 12.5µl SYBR Green qPCR SuperMix (including Taq DNA polymerase, SYBR Green I dye, Tris-HCl, KCl, 6mM $MgCl_2$, 400µM dGTP, 400µM dCTP, 400µM dATP, 800µM dUTP, uracil DNA glycosylase (UDG) and stabilizers, Invitrogen), 1.25µl forward primer (10µM, Invitrogen), 1.25µl reverse primer (10µM, Invitrogen), 1µl ROX fluorescent dye (Invitrogen) and 8µl nuclease-free water. Mastermix (24µl) was filled in 0.2ml 96-well plate (Thermo Scientific) followed by adding of 0.5µg cDNA as PCR templates. Wells were closed with strip caps, centrifuged and placed into PCR instrument (Stratagene Mx 3005P). The following PCR protocol was used: denaturation programme (95°C for 10 minutes), amplification programme repeated 40 cycles (denaturation: 95°C for 15 seconds, annealing: 60°C for 20 seconds, 72°C for 60 seconds with a single fluorescence measurement), dissociation programme (95°C for 60 seconds, 55°C for 30 seconds, 95°C for 30 seconds with a continuous fluorescence measurement) and a cooling programme to 40°C.

3.2.7 Statistical Analysis

For quantification of gene expression the cycle of threshold (Ct) for each gene transcript was determined. Ct is the point at which the fluorescence rises appropriately above the background fluorescence. The baseline signal and threshold signal of fluorescence were determined automatically by the PCR machine. Calculation was conducted in Microsoft Excel 2007. According to the delta delta-Ct method (Schmittgen and Livak, 2008), the relative expression ratio (RE) was calculated in relation to the expression of reference genes and was defined as relative expression of a target gene in a treatment sample versus a control:

$$\mathbf{RE=2\ ^\wedge\ (-(\Delta Ct_{sample}^{(target\text{-}reference)}-\Delta Ct_{control}^{(target\text{-}reference)}))};$$

$\mathbf{\Delta Ct_{sample}}$ is the Ct difference of target-reference genes in treatment samples;
$\mathbf{\Delta Ct_{control}}$ is the Ct difference of target-reference genes in PBS control.

Geometrical mean of Ct values of two reference genes were calculated to normalise target genes (Vandesompele et al., 2002). Mean and standard deviation (SD) of RE values were calculated from three replicates of each treatment. One-sample Student's t-test was performed in Graphpad Prism 5.0 to compare the difference of gene expression between the stimulated samples and PBS control, where the value of RE in PBS control was prescribed as 1.

4 Results

4.1 Optimisation of Primers and qRT-PCR

4.1.1 Purity of RNA and cDNA

Purity of RNA samples and cDNA templates was analysed by NanoDrop. For RNA samples, 260/280 values are > 1.8. 260/230 values range from 0.40 to 2.01. cDNA templates generated by reverse transcription from RNA samples have 260/280 values > 1.85 and 260/230 values > 1.2.

The ratio of absorbance at 260nm and 280nm is used for assessment of the purity of nucleic acids. It is generally accepted that a ratio 260/280 >1.8 indicates adequate purity. A reduced ratio may be caused by contamination with proteins or phenol that absorb strongly near 280nm. The ratio 260/230 is another measure of nucleic acid purity. Expected values of 260/230 range from 2.0 to 2.2, which is normally higher than the respective 260/280 values. A lower 260/230 ratio may indicate the presence of Ethylenediaminetetraacetic acid (EDTA), carbohydrates or phenol, which absorb near 230nm. However lower 260/230 do not interfere with the PCR reaction.

In my experiment, both RNA and cDNA samples had desirable 260/280 ratios but lower 260/230. As TRIzol, a phenol-based reagent, was used to extract RNA from cells, the low 260/230 might be due to the contamination from this phenol reagent. However, 260/280 values at high purity level denied TRIzol contamination. Therefore, other contaminants (eg. carbohydrates) with absorbance at 230nm rather than phenol might present. Furthermore, 280/260 values of RNA above 2.0 demonstrate no genomic DNA contamination. Thus the RNA sample was thought to be of

sufficient quality for reverse transcription. As all cDNA 260/280 above 1.85 theoretically exclude protein contamination, they were used as purified templates for qRT-PCR.

4.1.2 Optimisation of Reference Genes

When analysing results of qRT-PCR, several uncontrolled variables between samples can lead to misinterpretation of the results, such as different amounts of RNA and enzymatic efficiencies. In order to make a good comparison, normalisation to reference genes was used as a correction method for these variables. An adequate reference gene is expressed constantly at the same level throughout the experiment and between samples with different treatment. The geNorm analysis suggested GAPDH and B2M as two most stable reference genes under experimental conditions of the present study (Figure 1).

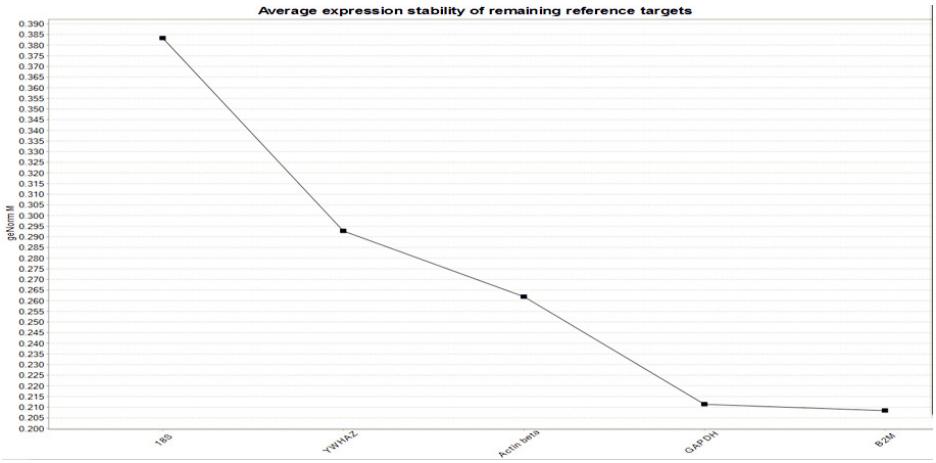

Figure 1: M view of geNorm analysis for five candidate genes. A ranking of candidate genes is shown from most unstable gene at the left to the best reference genes at the right (stability increases from left to right: 18S rRNA, YWHAZ, Actin beta, GAPDH and B2M).

4.1.3 PCR Specificity of Primers

4.1.3.1 Specificity of the First Set of Primers

With the first set of primers (Table 2), a few less sharp peaks appeared at low melting temperature before the final tall peak (green curves, Figure 2) in melting curves of both TREM2 (Figure 2A) and iNOS (Figure 2B) samples. Blunt peaks in melting curves of no template control (blue curves, Figure 2) indicate primer dimers,

and those of RNA control (red curves, Figure 2) indicate genomic DNA contamination. (No template control contained only primers without cDNA template; RNA control contained neither primers nor cDNA template, but only RNA samples without reverse transcription.)

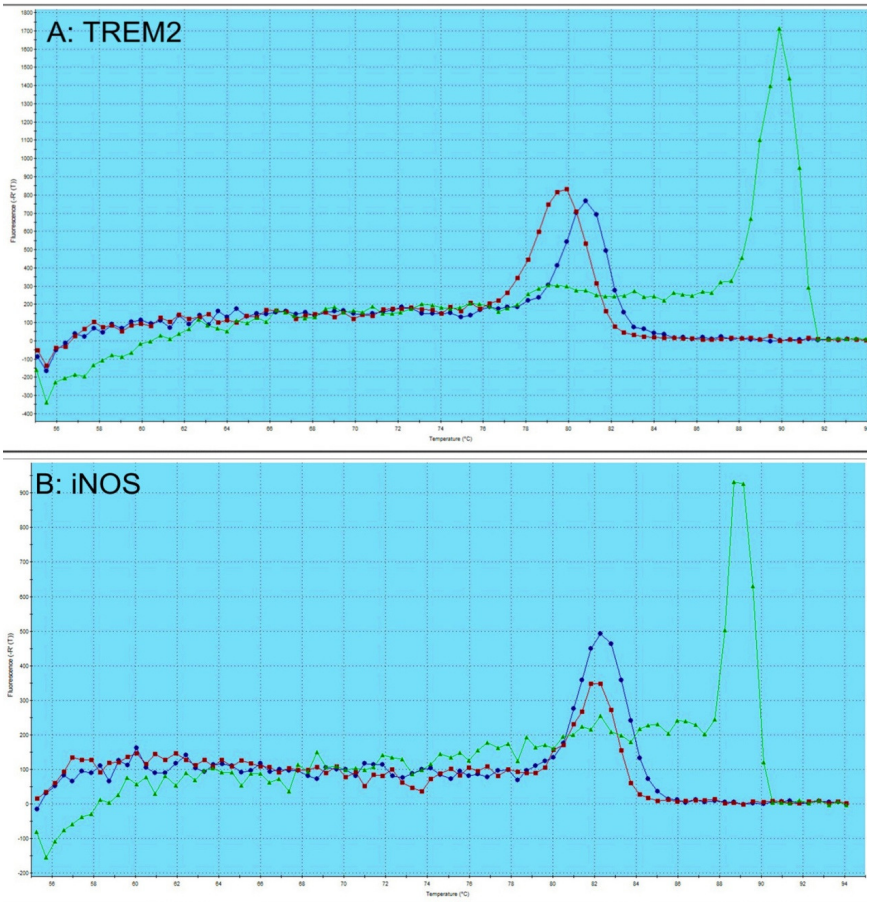

Figure 2: Melting curves of qRT-PCR for expression of TREM2 (A) and iNOS (B) in splenocytes. Blue curves: no template control; Red curves: RNA control; Green curves: samples with cDNA template.

These PCR products of iNOS were documented by gel electrophoresis (Figure 3), the result of which was in agreement with findings from PCR. Reading the gel from right to left, the second well was loaded with 100bp DNA Ladder. A smear of products with large base pairs appeared in well 4 for RNA control (red arrow), well 8 for no template control (blue arrow) and well 12 for iNOS-treated sample (yellow arrow). In addition to the smear band in iNOS sample, there was a second brighter band (green arrow) with less base pairs approximately at the position of the expected length (497bp) of iNOS.

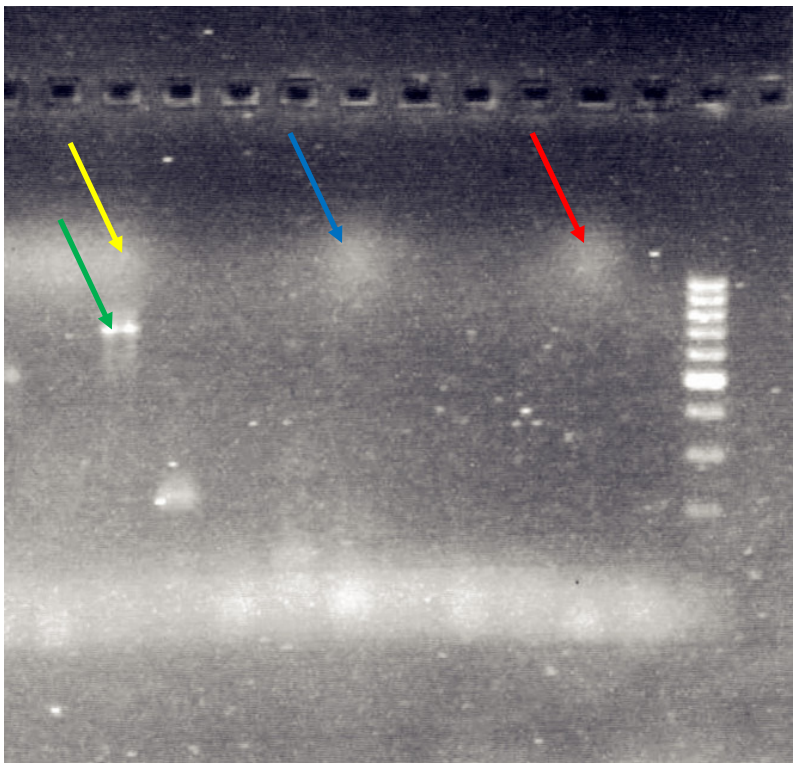

Figure 3: Electrophoresis of PCR products for iNOS expression in splenocytes. Yellow and green arrows: double-band products in iNOS samples; Red arrow: products in RNA control (no cDNA, no primer); Blue arrow: products in no template control (only primers, no cDNA template).

4.1.3.2 Specificity of the Second Set of Primers

To eliminate genomic DNA, DNase treatment was applied to RNA samples prior to the reverse transcription reaction and higher purity was obtained (260/280>2.0). To minimise primer dimer formation and improve PCR amplification efficiencies, sequences of primers were redesigned to generate a shorter product length. New primers were tested in BV2 cells and amplification specificity was proven by melting curves (Figure 4). The single distinct peak of melting curve for each gene indicates that a distinct single PCR product has been amplified.

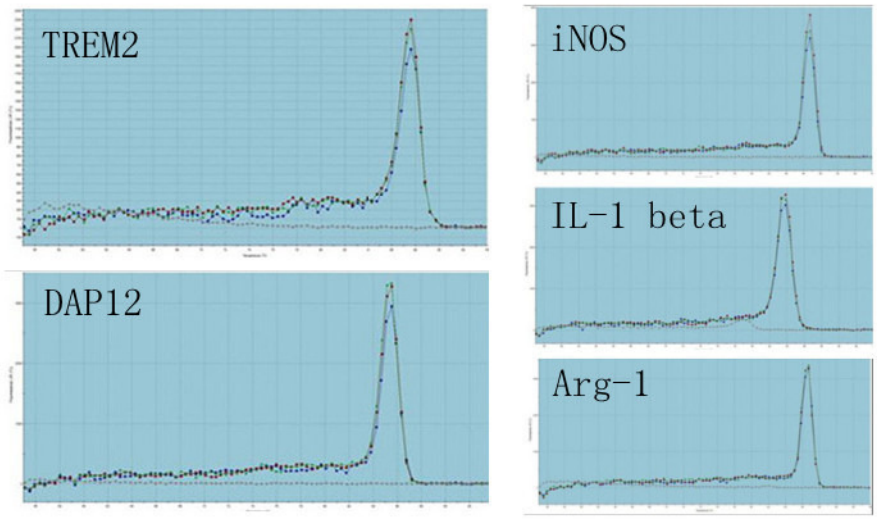

Figure 4: Melting curves of qRT-PCR for target genes with optimised primers. BV2 cells stimulated by LPS (red curves) or IL-4 (yellow curves) compared with PBS control (blue curves) and H_2O control (horizontal curves). The single peak in melting curves of each gene indicates high specificity of primers.

4.1.4 Efficiency of Real-time PCR

Efficiency of qRT-PCR was calculated from standard curves of serial dilution PCR of cDNA (Figure 5). All the investigated transcripts showed good efficiencies: TREM2, E=89.4%; DAP12, E=93.4%; iNOS, E=92.5%; IL-1β, E=95.3%; Arg-1, E=96.2%; GAPDH, E=95.2%; and B2M, E=94.4%. Improved efficiency is probably due to the shorter length of products of the second set of primers (Table 3) than of those in the first set (Table 2), which enabled better amplification in PCR.

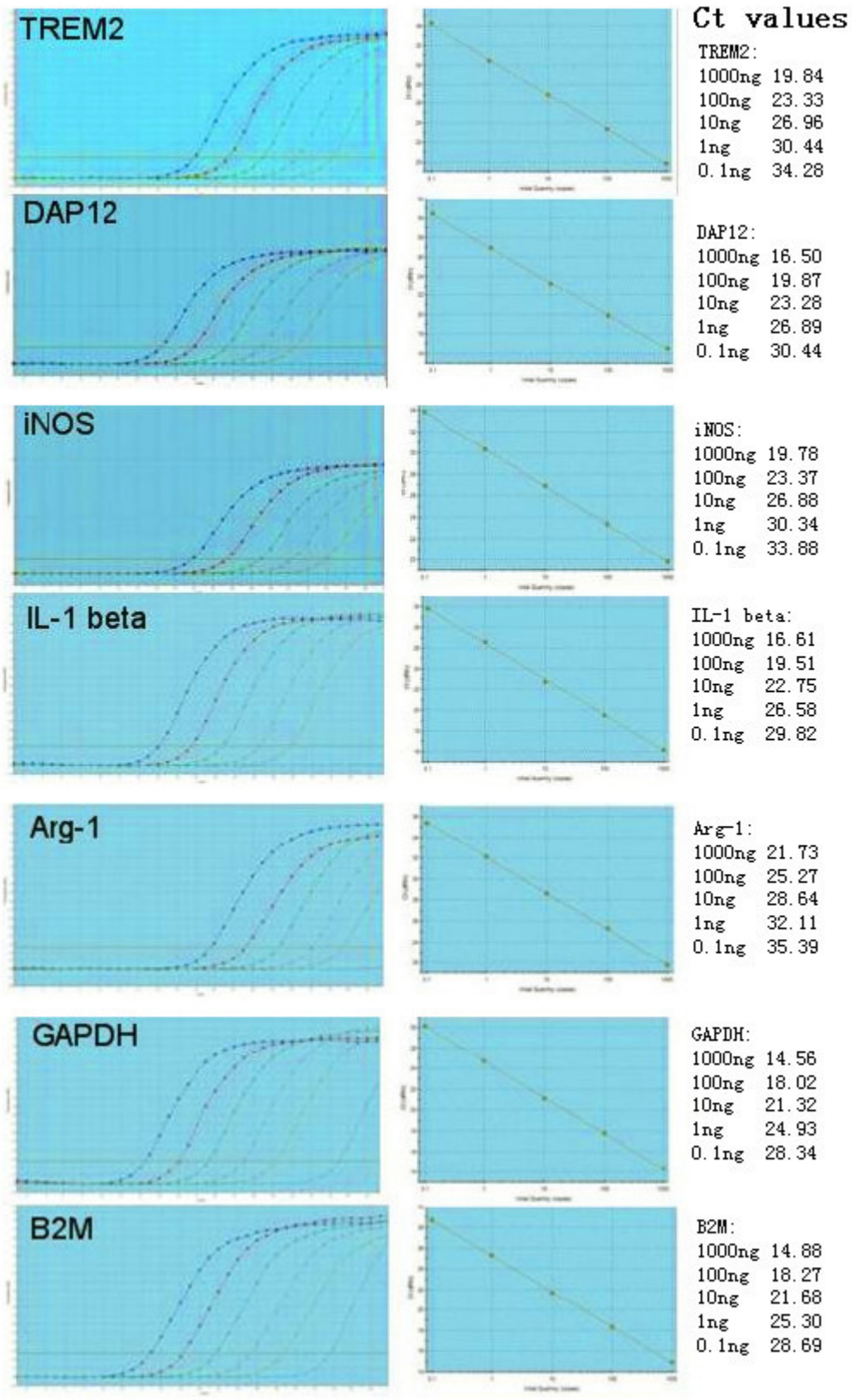

Figure 5: 10-fold serial dilution qRT-PCR for primer efficiencies. Graphs in the left column are amplification curves with diluted cDNA and those in the middle column are standard curves. The right column shows Ct values of each cDNA dilution for each gene.

As Ct values presented in amplification curves, Ct=20 occurred in samples with cDNA concentration ranged from 100ng/µl to 1000ng/µl. Therefore, 500ng/µl cDNA was used for later qRT-PCR analyses of target genes.

4.2 Constitutive Expression of TREM2 in BV2 Cells

To verify that BV2 cell constitutively express TREM2 and DAP12 and therefore provide a suitable system to study the regulation (up and down) of these genes, I performed immunocytochemistry. Expression of TREM2 (Figure 6) and DAP12 (Figure 7) in non-stimulated BV2 cells was clearly observed.

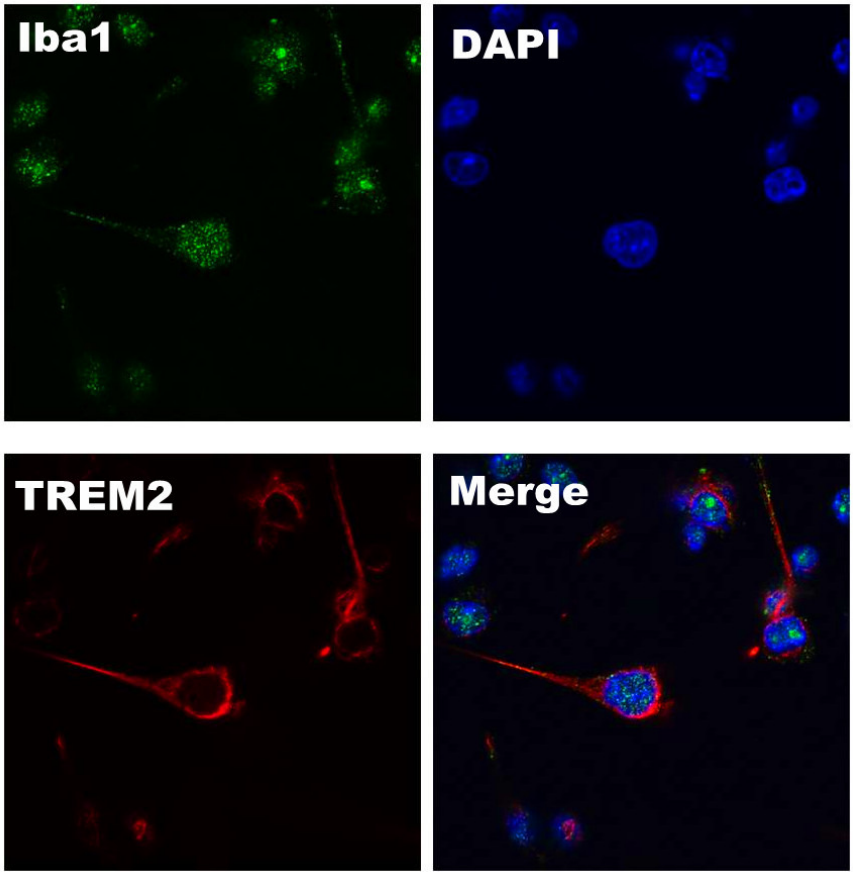

Figure 6: Immunocytochemistry of TREM2 expressed in unstimulated BV2 cells. The merged image exhibits TREM2 (red) with microglia marker Iba1 (green) and DAPI-stained nuclei (blue).

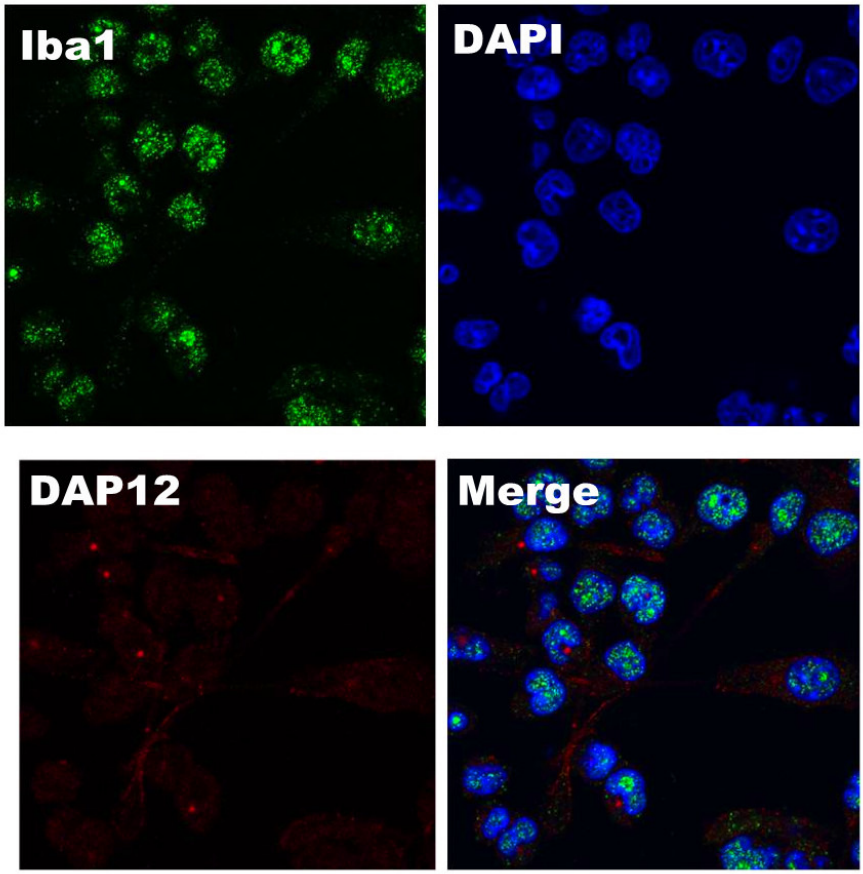

Figure 7: Immunocytochemistry of DAP12 expressed in unstimulated BV2 cells. The merged image exhibits DAP12 (red) with microglia marker Iba1 (green) and DAPI-stained nuclei (blue).

4.3 M1- and M2- stimulated TREM2/DAP12 Expression in Microglia

With all efficiencies are in range of 90% to 100%, the delta delta Ct method was adopted for relative quantification of PCR results. Relative expressions of genes are illustrated in Table 4. It should be noted that although statistical analysis has been carried out this is of an exploratory nature, because due to time constraints sample sizes (n = 3 independent cultures) are lower than needed to perform definitive comparisons in view of inter-culture variation. Therefore descriptions of changes in gene expression below are given with the above caveat in mind.

Table 4: Relative expressions (RE) of target genes. Mean and SD values of RE are calculated from three replicates of each treatment.

Target genes	Treatment		Relative Expression		
	Hours	Reagents	Mean	SD	P value
TREM2	6h	LPS	0.95	0.56	0.89
		IL-4	4.36	2.99	0.19
		HMGB1	4.56	4.20	0.28
	24h	LPS	0.40	0.07	<0.01
		IL-4	1.74	0.68	0.20
		HMGB1	1.20	0.20	0.22
DAP12	6h	LPS	0.55	0.20	0.06
		IL-4	0.70	0.24	0.17
		HMGB1	0.97	0.26	0.87
	24h	LPS	0.74	0.21	0.17
		IL-4	0.93	0.26	0.70
		HMGB1	1.25	0.58	0.54
iNOS	6h	LPS	1549.30	2093.78	0.33
		IL-4	44.96	33.50	0.15
		HMGB1	30.55	20.21	0.13
	24h	LPS	3318.13	2829.52	0.18
		IL-4	35.76	12.42	0.04
		HMGB1	26.45	13.56	0.08
IL-1β	6h	LPS	318.58	209.33	0.12
		IL-4	1.95	0.40	0.05
		HMGB1	1.11	0.58	0.77
	24h	LPS	207.82	152.89	0.14
		IL-4	2.90	1.64	0.18
		HMGB1	0.95	0.16	0.62
Arg-1	6h	LPS	0.87	0.34	0.57
		IL-4	159.91	90.92	0.09
		HMGB1	2.33	1.19	0.19
	24h	LPS	0.60	0.27	0.13
		IL-4	591.20	669.65	0.27
		HMGB1	1.35	0.70	0.48

4.3.1 Expression of Inflammatory Mediators under M1 and M2 stimulation

The expected polarisation toward M1 and M2 activation phenotypes by LPS and IL-4 respectively were confirmed in BV2 microglial cells. Expression of both iNOS (Figure 8A, 8B) and IL-1β (Figure 8C, 8D) was markedly increased in LPS samples, whereas Arg-1 expression was unaffected (Figure 8E, 8F). In contrast, a marked increase in Arg-1 expression with relatively modest increases in iNOS and IL-1β were observed in IL-4 –treated samples. HMGB1 stimulation produced no clear polarisation toward M1 or M2 phenotype, although an increase in iNOS expression is apparent.

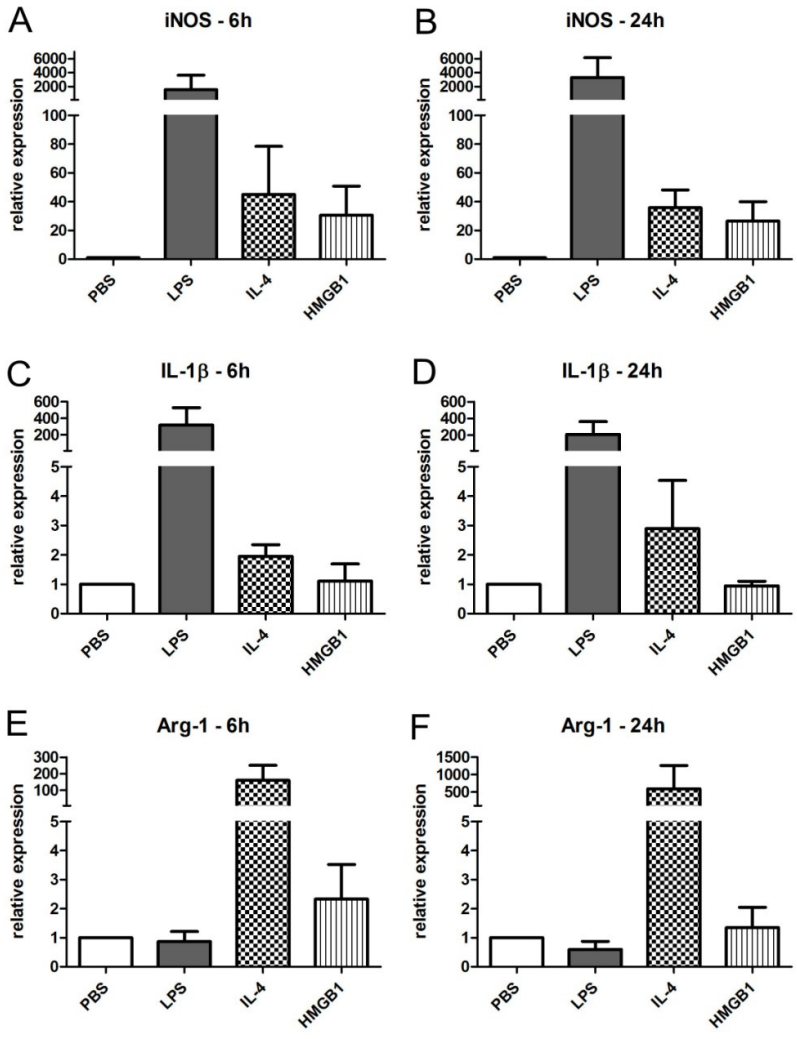

Figure 8: Relative expression of iNOS, IL-1β and Arg-1 in BV2 cells stimulated by LPS, IL-4 or HMGB1. A: iNOS expression by 6-hour stimulation; B: iNOS expression by 24-hour stimulation; C: IL-1β expression by 6-hour stimulation; D: IL-1β expression by 24-hour stimulation; E: Arg-1 expression by 6-hour stimulation; F: Arg-1 expression by 24-hour stimulation.

4.3.2 Microglial TREM2 but Not DAP12 Expression is Regulated by M1 and M2 Polarising Stimuli

TREM2 expression (Figure 9A, 9B) in LPS stimulated BV2 cells was suppressed after 24h but not 6h, but showed a trend towards an increase in expression by IL-4 after 6h. DAP12 (Figure 9C, 9D) presented no obvious difference between treatments.

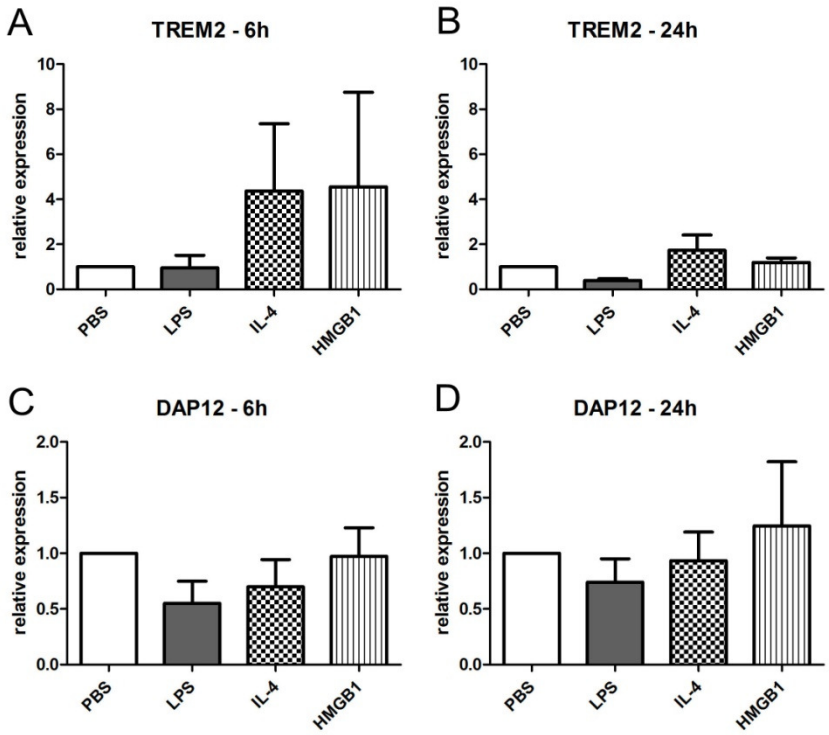

Table 9: Relative expression of TREM2 and DAP12 in BV2 cells stimulated by LPS, IL-4 or HMGB1. A: TREM2 expression by 6-hour stimulation; B: TREM2 expression by 24-hour stimulation; C: DAP12 expression by 6-hour stimulation; D: DAP12 expression by 24-hour stimulation.

By one-sample student's t-test, 95% confidence interval (95% CI) of RE in all samples (except one, TREM2 expression in 24-hour LPS-stimulated sample) crossed 1 and P values were greater than 0.05. However, it was difficult to draw a definite conclusion that there was no difference between samples and control, because within the time allocated for the project it was possible to include only three replicates for each treatment group.

5 Discussion

5.1 Validation of M1- and M2-activation in Microglia

In BV2 microglia cells, iNOS was up regulated while Arg-1 was unaffected in LPS-treated samples. Though IL-4 also promoted a more modest induction of iNOS, Arg-1 expression was strongly induced in IL-4-treated samples. It is tempting to speculate that IL-4 -activated BV2 cells are not adept at killing microorganisms though they produce some iNOS, but rather their primary role is in tissue repair. In addition, iNOS expression was stable from 6h- to 24h-stimulation by IL-4, but Arg-1 exhibited a five-fold increased expression with extended stimulation. It further indicates IL-4 as a positive regulator for Arg-1 expression. These finding are in agreement with previous studies with macrophages, in which M1 macrophages were activated by LPS and contributed to microbial killing and IL-4 induced M2 macrophages potentiated Arg-1 expression and participated in tissue repair (Gordon, 2003; Hesse et al., 2001).

IL-1β is a potent pro-inflammatory cytokine in responses to infection and inflammation. Macrophages are major peripheral sources of IL-1 and microglia are cells producing it in the CNS (van Dam et al., 1992). In both in vitro and in vivo studies, IL-1β was up regulated by LPS (Lee et al., 1993; Van Dam et al., 1995). This property was confirmed by my work, in which expression of IL-1β was three hundred times higher in LPS-treated samples compared with vehicle controls. As a pro-inflammatory mediator, IL-1β exhibits a similar of induced expression to iNOS. A slight elevation of IL-1β expression was observed in IL-4-stimulated samples and an even minor increase HMGB1 samples.

Collectively, these data suggest that BV2 microglia adopt similar phenotypes to macrophages in response to M1 and M2 activating stimuli.

5.2 Association of M2 Phenotype with Activation and M1 Phenotype with Suppression of Microglial TREM2 Expression

Since microglia are a subtype of macrophages, I anticipated that microglial expression of TREM2 may be consistent with M1 and M2 activation patterns observed in macrophages. In this study, M1 activation with LPS and M2 activation

with IL-4 in BV2 microglial cells have been well established by the expression of iNOS and Arg-1. Therefore, the expression of TREM2 and DAP12 in microglia is discussed with reference to stimulation by these two reagents. A high level of expression of TREM2 by non-stimulated BV2 cells was demonstrated by immunocytochemistry analysis (Figure 6). The expression was down regulated by LPS and up regulated by IL-4. The reduced TREM2 expression in microglia by LPS was supported by the work of Schmid (Schmid et al., 2002). It is also consistent with another study of macrophages in which LPS abrogated and IL-4 promoted the expression of TREM2 (Turnbull et al., 2006). Therefore, the association of microglial TREM2 expression with M2 activation and its inhibition by M1 signals mirrors the pattern observed in macrophages.

5.3 Stable Expression of DAP12

Unlike TREM2 regulated by LPS and IL-4, no obvious difference in DAP12 expression were observed between PBS controls and stimulated samples. A possible explanation is that DAP12 serves as an adaptor for both TREM1 and TREM2, which have contrary immune effects. TREM1 is a pro-inflammatory receptor and is up regulated by LPS, whereas TREM2 is, in contrast, anti-inflammatory and is down regulated by LPS (Bouchon et al., 2000). In an earlier mouse study (Kiialainen et al., 2005), expression patterns of TREM2 and DAP12 during the CNS development were similar and even presented both temporal and spatial co-localization, however, DAP12 expression exceeded that of TREM2. The high constitutive expression of DAP12 and its less inducible property under inflammatory stimulation may reflect its role as an adaptor for both positive and negative regulators of inflammatory responses.

5.4 TREM2/DAP12 Signalling in Microglia

For each gene, expression in all samples was broadly consistent between 6-hour and 24-hour stimulation. LPS-induced down-regulation of TREM2 co-existed with increased expression of iNOS and IL-1β. IL-4 promoted TREM2 expression was accompanied by enhanced production of Arg-1. The converse expression of iNOS and

Arg-1 appear to define two alternate functional states of microglia, involving M1 and M2 mediators, respectively.

Molecules and mechanisms involved in TREM2/DAP12 dowmstream signalling may provide targets for regulation of TREM2 effects on microglia. ITAM-mediated DAP12 signalling has been well established in NK cells (McVicar et al., 1998). It is reported that phosporylated ITAMs of DAP12 provide docking sites for tyrosine kinase ζ-chain associated protein 70 (ZAP70) and spleen tyrosine kinase (Syk), eliciting subsequent kinases cascades to activate p44/p42 extracellular-signal regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ1 (PLCγ) pathways. These signals ultimately lead to intracellular Ca^{2+} mobilization, reorganisation of cytoskeleton and activation of transcription of immune mediators (Colonna, 2003).

Similar DAP12 downstream signalling has been characterised in DCs and macrophages. As several downstream pathways are available for DAP12 signalling, it is important to work out certain molecules participating in TREM2/ DAP12-mdediated cellular activities. ERK was reported to get involved in TREM2-triggered activation of DCs (Bouchon et al., 2001), however, whether TREM2-DAP12-ERK pathway occurs in macrophages is controversial. Hamerman *et al*. (Hamerman et al., 2005) reported increased activation of ERK in DAP12 knockdown macrophages in response to LPS, while such activation was not observed in a study of TREM2 knockdown cells (Turnbull et al., 2006). Conversely, an earlier study demonstrated that transduced stimulation of TREM2 in microglia induced phosphorylation of ERK (Takahashi et al., 2005). Thus, more research is needed to clarify the role of ERK in macrophage/microglia TREM2-DAP12 signalling. TREM-2 was reported to inhibit TLR- and FcR-induced macrophage responses (Hamerman et al., 2006). Another intracellular messenger involved in DAP12 signalling, PI3K, suppresses TLR activation (Fukao and Koyasu, 2003). Therefore, TREM2-DAP12-PI3K pathway may account for this inhibitory effect on macrophages, which down regulates TLR-mediated expression of iNOS and IL-1β. But it is interesting that, in my experiment, increased secretion of IL-1β occurred with both promotion and suppression of TREM2 expression; whereas in a previous study, no difference of IL-1β expression was

identified between wild type microglia and those with TREM2 transduction (Takahashi et al., 2005). Therefore, such mechanisms are to be further investigated.

In addition, expressions of iNOS and IL-1β shared a similar pattern upon different stimulation, which was significantly enhanced by LPS and moderately promoted by IL-4. Therefore, I speculated the presence of an association between iNOS and IL-1β. It is reported that IL-1β induced iNOS expression via NF-κB in astrocytes, another type of glial cells, and a NF-κB inhibitor suppressed iNOS production (Chao et al., 1997). To study whether such mechanism could be applied to microglia, future work could be devoted to iNOS expression in both IL-1β-activated and inhibited microglia, and also by suppression of molecules involved in IL-1β/NF-κB signalling pathway.

5.5 Clinical Perspectives: Microglial TREM2 and CNS Homeostasis

Dysfunction of microglia is proposed to be a feature in neurodegenerative diseases. Hsieh *et al*. (Hsieh et al., 2009) identified certain ligands on neurons, which associated with TREM2 expressed on microglia to elucidate the removal of apoptotic neurons. Therefore, TREM2-mediated microglial phagocytosis may be essential for maintaining or restoring CNS homeostasis during development, ageing and pathology. Microglia eliminate tissue debris and dead cells therefore can minimise brain inflammatory changes with the potential to cause neuronal damage. However, microglia also play a detrimental role in brain diseases by promoting inflammation and activated microglia with overexpression of immune mediators could potentiate brain injury.

In neuropathologies inflammation occurs as a local response initiated in part by microglia. It is controversial whether microglial activation and brain inflammation are beneficial or detrimental. On the one hand, activation of microglial phagocytosis without excessive cytokines production is important for surveillance of the CNS. On the other hand, activation of microglia is characteristic to most neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease and brain ischaemia (Carson et al., 2002; Dickson et al., 1993; Morioka et al., 1993). Pro-inflammatory cytokines IL-1β and TNFα secreted by activated microglia are potent mediators of neuronal

death in animal models of neurodegenerative diseases. Takahashi *et al.* (Takahashi et al., 2005) reported that TREM2-stimulated microglia had increased phagocytosis and retarded inflammatory activities. In his later work with experimental autoimmune encephalomyelitis (EAE) (Takahashi et al., 2007), an animal model of multiple sclerosis, intravenous transfusion of TREM2-transduced myeloid cells alleviated clinical symptoms in EAE-diseased mice. Therefore, TREM2 is a potential target for attenuation of microglia-mediated brain inflammation. In Alzheimer's disease, microglia express cytokines, enzymes and receptors which are involved in degradation and phagocytosis of amyloid plaques. However, immune mediators secreted by microglia might also contribute to inflammatory impairment to brain. As TREM2 of microglia fulfils important functions in tissue debris clearance and resolution of inflammatory reactions, it might retard pathological processes of Alzheimer's disease. Regulation of microglial TREM2 is also promising for brain ischaemia. It is implicated that inflammation is a secondary mechanism of brain injury following ischaemia. The microglial responses to ischaemic injury may be either beneficial by eliminating brain tissue debris or harmful by facilitating neuronal death. If regulation of microglia through TREM2 signalling pathway could be achieved, it will help attenuate brain inflammation and enhance tissue repair after ischaemic insults.

There are several approaches to regulate TREM2-mediated immune activities of microglia. Genetic modification is a most direct intervention to regulate TREM2 expression but its clinical application is limited by current technologies. The M1 and M2 polarising theory proposed in current study provide a more realistic strategy. It targets molecules involved in LPS and IL-4 activation of TREM2 expression. Although current work suggested that, in general, promoting an M2 environment is in favour of TREM2 expression, it remains unclear what signalling cascades and transcription factors are involved in M1 and M2 regulation of TREM2. Future work is expected to clarify such mechanisms. HMGB1-mediated DAMP-associated pathway might be another mechanism for TREM2 regulation, but such effects were not well illustrated due to insufficient data in this experiment. A third approach is to target TREM2 ligands. Though have yet to be fully identified, certain anionic ligands identified in

bacteria and astrocytes associate to TREM2 via a charge-dependent interaction (Daws et al., 2003) and Heat Shock Protein 60 (HSP60) is another TREM2 ligand expressed on surface of neurons and astrocytes (Stefano et al., 2009). Mimic proteins of such ligands might play a role as TREM2 agonists.

5.6 Limitations and Future Prospective

This is the first study relating TREM2 expression in microglial cell line to M1 and M2 types of inflammatory stimulation. This study has been constrained by the 12-week project period and most time has been devoted to optimise the experimental approaches. Should more time be allowed, sufficient data could be generated to provide more consistent and definitive results, in particular by increasing the number of replicates. However, this study still provides some valuable information for future studies, such as stable reference genes and high efficiency primers for related target genes. In addition, although the statistical analysis must be interpreted with some caution, the experiment has revealed a trend of inflammatory regulation of TREM2 expression and other immune responses in microglia. Further studies with a large sample of replicates and *in vivo* analysis may help to provide more detailed and reliable evidence to reveal regulation of microglial TREM2.

Using an immortalised cell line instead of primary cells is another potential limitation of this study. Although BV2 cells recapitulate many properties of microglia, such as phagocytosis, they fail to model primary microglia in production of certain cytokines under certain stimulation. IL-1β is one of these cytokines that expressed different by activated BV2 cells and primary microglia. Horvath *et al.* (Horvath et al., 2008) reported that BV2 cells constitutively secreted IL-1β whereas primary microglia did not. It might be a possible explanation for a relatively lower expression of IL-1β compared to iNOS in LPS samples of the present work. Since BV2 cell line only partially models primary microglia, it will be important to replicate these data in primary microglia and also *in vivo*.

6 Conclusion

Generally, BV2 microglial cells exhibit similar immune properties to macrophages following M1 and M2 inflammatory stimulation, respectively. The M2-polarising cytokine IL-4 induces up regulation of TREM2 and Arg-1, which are potentially important to attenuate brain inflammation and facilitate tissue repair. The M1 activating reagent LPS stimulates production of pro-inflammatory mediators iNOS and IL-1β, and inhibits the expression of Arg-1 and TREM2. Regulation of microglial TREM2, particularly in the context of an environment favouring M2 polarisation, provides a promising target for interventions in neurodegenerative diseases with inflammatory pathology.

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